MAITOTOXIN INDUCES MUSCLE CONTRACTION AND A NON-SELECTIVE CATIONIC CURRENT IN SINGLE SMOOTH MUSCLE CELLS OF THE GUINEA-PIG PROXIMAL COLON

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We have investigated the mechanisms of action of maitotoxin-2 (MTX), a marine toxin isolated from the toxic dinoflagellate, *Gambierdiscus toxicus*, on the contractility of the intact circular smooth muscle of guinea-pig proximal colon and on the membrane currents recorded in enzymatically-dispersed single cells from this muscle, using standard contraction and patch clamp recording techniques. MTX (0.005-5.0nM) induced an initial phasic contraction and a subsequent cessation of all spontaneous contractile activity, which was sometimes associated with a sustained increase in muscle tone. The initial contraction to MTX was blocked by atropine (2μM), the muscarinic receptor antagonist. Contractions to acetylcholine (0.5μM) were reduced approximately 75% after 7 minutes exposure to MTX (0.5nM) (n=5), this blockade was resistant to washout. MTX (5nM) completely abolished the contractions to acetylcholine, but reduced contractions to raised external concentrations of K^+(40mM) only 54 ±9% (n=4).

Single colonic smooth muscle cells were perfused with K^+- filled patch pipettes and voltage clamped at a holding potential of -80 mV. MTX (5nM) added to the bathing solution induced a large inward current (1-3 nA) after 15-45 minutes. Development of this current was not prevented by a number of K^+ channel blockers, including tetraethylammonium (TEA; 2–126 mM), 4-aminopyridine (5mM), quinidine (5mM) or glibenclamide (10μM); nor when the Ca^{2+} was removed, or replaced with Ba^{2+} (7.5mM). This current was, however, blocked by Cd^{2+} (0.1-1mM) and reduced by nifedipine (10μM) and La^{3+} (1mM). The MTX-activated current had an almost linear current-voltage relationship with a reversal potential near –30 and 0 mV when cells were respectively filled with K^+ or Cs^+. When most of the extracellular Na^+ (126mM) was replaced with TEA^+, this current reversed near –60mV. These results suggest that MTX induces the appearance/opening of voltage-insensitive channels which allow the flow of Na^+, K^+ and Cs^+, but not TEA^+, and which are blocked by Cd^{2+}.

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Ciguatoxins(CTX) and maitotoxins(MTX) are potent marine toxins isolated from the dinoflagellate *Gambierdiscus toxicus*. Ciguatoxins are lipid soluble and accumulate in the flesh and viscera of reef fish; they are the principal toxins responsible for cigatera. Maitotoxins are more polar and extracted in a number of forms from cultures of *G. toxicus* (Yokayama et al.,1988; Holmes et al., 1990). They have positive inotropic effects on cardiac muscle (Kobayashi et al.,1985) and cause contraction in smooth muscle (Ohizumi & Yasumoto,1983). They also induce a rise in the internal Ca^{2+} levels in BC_3H_1 muscle cells (Sladeczek et al.,1988) and aortic smooth muscle cells in culture (Berta et al.,1988) associated with phosphoinositide metabolism (Gusovsky et al., 1987,1988; Sladeczek et al.,1988; Meucci et al., 1992), and promote release of transmitters from neurones and hormones from a number of secretory cells (Kim et al.,1985; Gusovsky et al., 1988). Almost all of these effects of MTX depend on the presence of extracellular Ca^{2+} and can be, depending on the tissue, blocked by both organic (verapamil, some dihydroprydines) and inorganic Ca^{2+}-channel entry blockers (Cd^{2+}, Ni^{2+} and Co^{2+}). This rise in intracellular Ca^{2+} induced by MTX has therefore been suggested to arise from (*i*) modulation of voltage-activated Ca^{2+} channels (Kobayashi et al.,1987, Yokayama et al.,1988), (*ii*) the mobilization of Ca^{2+} from internal stores (Meucci et al.,1992), or (*iii*) from the influx of Ca^{2+} through MTX-activated pores or channels (Yoshii et al.,1987; Sladeczek et al., 1988). Here, we describe that MTX-2 inhibited

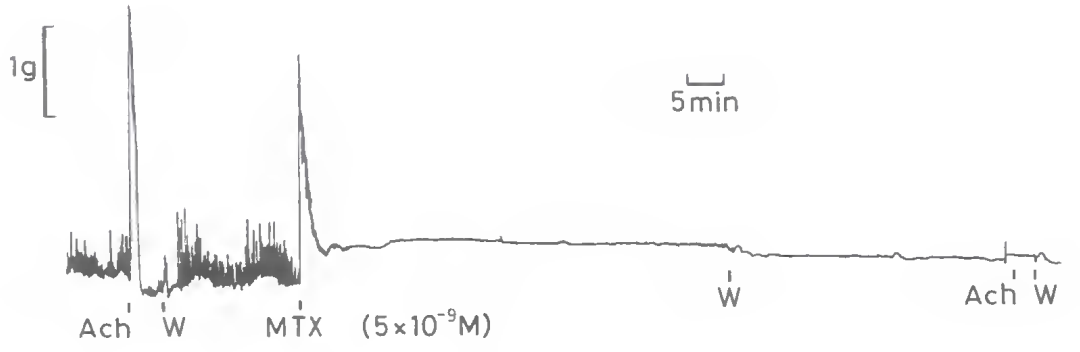

FIG. 1. Effects of acetylcholine and MTX-2 on the contractile activity of circular muscle strips of the guinea-pig proximal colon. MTX (5nM) induced a transient contraction sensitive to atropine (2μM). Contractions to acetylcholine (Ach, 5μM) were abolished irreversibly, even after extensive washout (W) of MTX.

the spontaneous and acetylcholine-induced contractile activity in the intact guinea-pig proximal colon. These effects are likely to be related to the large inward current observed in voltage clamped single cells exposed to MTX, arising from the induction of MTX channels selective for K^+ and Na^+. Some of these results have been presented previously in brief (Lang et al.,1992).

METHODS

MTX-2 used in this study was isolated from cultures of the NQ1 strain of *G. toxicus* and purified to homogeneity on HPLC as previously described (Holmes et al.,1990). This MTX has a molecular weight of 3290 Daltons for the sodium salt, a LD_{50} of 0.08μg/kg in mice (applied intraperitoneally) and was dissolved in a small volume of methanol: water (1:1).

Contraction Studies

The proximal colon (6cm long, 1–2cm aboral of the caecum) of the guinea-pig was excised and cut into 0.5cm rings Preparations were placed in 5ml organ baths and suspended under 0.5g tension between two stainless steel rods to allow recording of circular muscle contraction.

Isometric recordings (F-60 Narco Biosystems) were made at 37°C. Tissues were maintained in oxygenated (95% O_2: 5% CO_2) physiological saline solution containing (mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 1.0; KH_2PO_4 0.5, $NaHCO_3$ 11.9; glucose 5.5. Preparations were equilibrated for 30 min and then used after obtaining reproducible responses to acetylcholine (5μM).

Cell Dissociation

The proximal colon (3–4cm long), 1–2cm aboral of the caecum, of the guinea pig was excised, cut open longitudinally and pinned out, mucosal surface uppermost, in a dissecting dish filled with a nominally Ca^{2+}-free physiological saline (PS)(see below). After removal of the mucosa, the circular muscle layer was peeled from the underlying longitudinal layer, cut into small pieces ($2mm^2$) and rinsed in low-Ca^{2+} (30 μM) PS for 2 min (at 37°C). The muscle pieces were then transferred to low-Ca^{2+} PS containing: collagenase Type 1(0.6mg/ml; Worthington); bovine serum albumin (2mg/ml; Sigma) and trypsin inhibitor (0.2mg/ml; Sigma). After a 60 minute incubation period, the muscle pieces were re-suspended in low-Ca^{2+} PS and gently agitated for 10 min (at 37°C). Single cells were obtained by gentle trituration with a wide-bore glass pipette. Cells were allowed to settle for 5-10 min to the glass bottom of the recording chamber mounted on an inverted microscope; the solution was then exchanged for normal Ca^{2+} (1.5mM) PS (Vogalis et al.,1993).

Whole-cell and Single Channel Current Recordings

Patch pipettes were drawn from glass capillary tubing (1.5–1.8mm; Kimax-51, Kimble, USA) on a programmable micro-pipette puller (Sachs-Flaming PC-84, Sutter Instruments) and their tips fire polished (MF-84 Narishige). Pipettes resistances ranged from 2–7MΩ when filled with pipette solution. Single channel and whole-cell membrane currents were recorded at room temperature using an Axopatch 200 (Axon In-

struments) and conventional patch-clamp techniques (Hamill et al.,1980). Current and voltage signals from the patch-clamp amplifier were digitized with a Labmaster TM125 analog-to-digital device (Scientific Solutions) interfaced to an Arrow-AT desktop computer using p-CLAMP software (Axon Instruments). Digitized data were stored and analyzed using this p-CLAMP software.

The physiological saline (PS) was of the following composition (mM): NaCl 126; KCl 6; HEPES 6; d-glucose 11; $MgCl_2$ 1.2; $CaCl_2$ 1.5; adjusted to pH 7.4 with 5M NaOH. The pipette solution contained (mM): KCl 126; HEPES 6; Na_2ATP 3; EGTA 3; $MgCl_2$ 3; d-glucose 11; pH was adjusted to 7.4 with 5M KOH. In some cells, current flow through all K^+ channels was blocked by replacing the KCl in the pipette solution with an equimolar concentration of CsCl, pH was set with 5M NaOH (Vogalis et al.,1993).

RESULTS

Contraction Studies

A typical isometric recording of the spontaneous contractions in a strip of circular muscle from the guinea pig proximal colon is illustrated in Fig.1. Acetylcholine (Ach, 5μm) caused a transient increase in muscle tension. MTX (5nM) produced a similar transient increase in tension which was sometimes followed by a maintained increase in muscle tone associated with a loss of the spontaneous contractile activity. This increase in muscle tone decreased upon washout (W) of the MTX, the spontaneous contractile activity, however, did not return. These effects of MTX were concentration dependent. Threshold phasic contractions (0.07-0.22g) to MTX were evoked with concentrations between 0.005 and 0.5nM MTX (n=3-5), substantial contractions (>2g) were only recorded with 5 nM MTX (n=7). In four muscle strips, these effects of MTX (5nM) were blocked by atropine (2μM), the muscarinic receptor antagonist.

The contractions to Ach (5μM) were completely abolished by MTX (5nM) and never recovered, even 145 minutes after the removal of MTX (n=6). In contrast, contractions elicited by direct muscle depolarization with 40mM K saline were decreased only 54.6±8.7% (n=4) by MTX (5nM). These effects of MTX were also concentration dependent, the concentration of MTX which half-maximally inhibited the Ach contractions was approximately 0.2nM MTX. These effects of MTX were mimicked in part by monensin, the Na^+ ionophore, which blocked the acetylcholine contractions dose-dependently (0.1–10μM), half-maximal reduction was achieved with approximately 0.2μM monensin (n=4). Monensin, however, did not induce any muscle contraction. These data suggest that MTX, monensin and acetylcholine may well be sharing a common mechanism of action. In view of this, the experiments below describe our preliminary investigations of the action of MTX on the membrane channel currents recorded in single smooth muscle cells of the guinea-pig proximal colon.

Control Whole-cell Current Recordings

Resting membrane potentials of –40 to –60 mV were recorded when a 6mM: 126 mM K^+ gradient was established across the cell membrane of single cells of the proximal colon. Depolarizing currents triggered action potentials which peaked between –10 and 0mV and had durations of 100-200ms at their half-maximal amplitude. Under voltage clamp, depolarizations, from a holding potential of –80mV, triggered complex membrane current responses (Fig. 2A). At potentials positive to –60mV, a rapidly-activating and inactivating outward current was triggered. At more positive potentials (–20mV) the transient current, in most cells, was followed by a second slowly developing and decaying outward current. The pharmacological identification of three K^+-channel currents and one Ca^{2+}-channel current underlying these current responses to membrane depolarization has been demonstrated (Vogalis et al.,1993) and will be briefly summarized.

A substantial portion of the second slowly-decaying outward current recruited at positive potentials is blocked by the addition of tetraethylammonium (TEA) (2–5mM) and a Ca^{2+}- entry blocker (0.1 mM Cd^{2+}) to the bathing solution, suggesting that this current flows through the large conductance Ca^{2+}-activated ('maxi' or 'BK') K^+ channels (I_{KCa}) which have been recorded in all smooth muscles so far examined (Vogalis et al.,1993). The Ca^{2+}-insensitive current remaining in the presence of low TEA (2–5mM): Cd^{2+} (0.1mM) activates rapidly and then decays slowly to a sustained current after 400ms, and can be further divided into two K^+ channel current components. A slowly-activating, non-inactivating K^+ current (I_{Kdel}), which has characteristics similar to delayed rectifier K^+ currents found in many electrophysiological preparations (Rudy,1988), is revealed when 4-aminopyridine (4-AP) (5 mM) blocked the initial

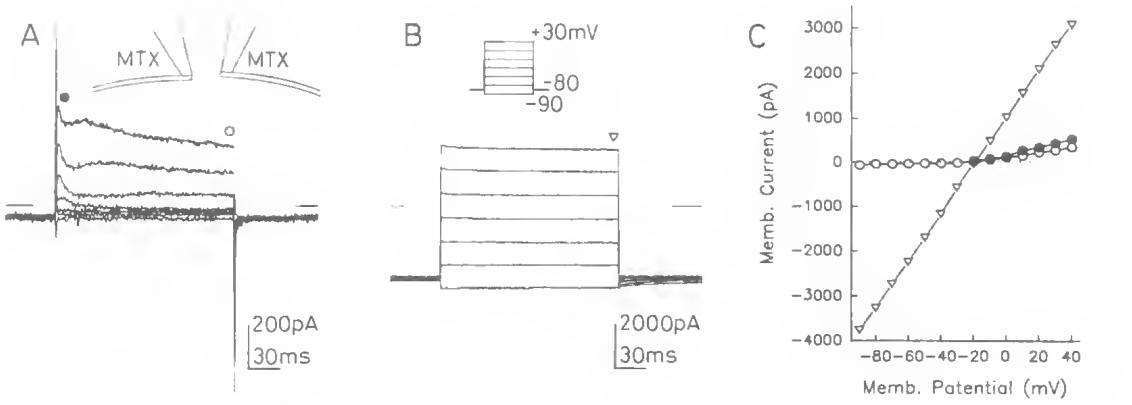

FIG. 2. Effects of MTX (5nM) on the whole-cell membrane currents recorded in single cells of the guinea-pig proximal colon. Cells were voltage clamped at a holding potential of -80mV with a K^+-containing pipette. Stepped changes in potential (100-400ms in duration) were applied to cells to evoke voltage-gated membrane channel currents in control solutions (A) and 34 minutes after exposure to MTX (5nM)(B); short lines on either side of these panels represent the zero current level. Note the ten-fold change in the vertical scale in B. C, current voltage plots of the initial peak amplitude (filled circles) in control saline and the end of pulse current before (hollow circles) and after MTX exposure (filled triangles).

itial transient component of the Ca^{2+}-insensitive K^+ current. On the other hand, blockade of the sustained component of the Ca^{2+}-insensitive current with TEA (12–20mM) reveals the time course of the rapidly activating and inactivating transient outward current, (I_{Kto}) which was blocked by 4-AP (5mM) (Rudy,1988). In cells recorded with pipettes containing Cs-saline, stepped depolarizations elicits an inward current at potentials positive to –40mV which peaks near +10mV, reverses in direction near +50mV and decays slowly over 400ms. This inward current was increased when Ba^{2+} replaced Ca^{2+} in the bathing solution and blocked by the Ca^{2+}-entry blockers, nifedipine (10μM) or Cd^{2+} (0.1mM), indicating that it represents current flow through 'high-voltage activated' or 'L-type' Ca^{2+} channels (I_{Ca}) (Vogalis et al.,1993).

Action of Externally-applied MTX

MTX (5nM) induced a massive increase in the holding current (at a holding potential of –80 mV), from about 50–100pA to about 1000-3000 pA, but only after a delay of some 15–45 minutes (n=14 cells). When the whole-cell membrane currents (elicited every 20mV between –90 and +30mV) recorded before (Fig.2A) and 34 minutes after (Fig.2B) the application of MTX (5 nM) are illustrated, note the ten-fold change in the vertical scale in Fig.2B. The time-dependent whole-cell currents in control saline were totally swamped by the MTX-induced current (I_{MTX}) which shows little time dependence. During the development of this inward current, however, MTX had little effect on the three whole cell K^+ channel currents or on the Ca^{2+} channel current which underlie the currents in Fig.2A (data not shown). In fact, development of I_{MTX} was little affected by a number of known K^+ channel blockers such as TEA (2–126mM), quinidine (0.5 mM), 4-AP (5mM), glybenclamide (10–20μM) or Ba^{2+} (7.5mM).

The current-voltage (I-V) relationship of the initial peak amplitude (I_{Kto}) and the current at the end of these depolarizing steps in control saline (I_{KCa} + I_{Kdel}) and 34min after MTX (5nM) (Fig.2C) shows that I_{MTX} has no voltage dependence (linear) and reverses in direction near –20 mV, suggesting that MTX induces an increase in the membrane conductance to the cations Na^+ and K^+, or to Cl^-, possibly by the opening of membrane channels.

The ionic selectivity of these MTX-activated channels was investigated by substituting the extracellular Na^+ and intracellular K^+ with other cations known to have different permeating properties. In the TEA^+ (126mM) saline, I_{MTX} induced by 5nM MTX (Fig.3A,top panel; B) was linear between –90 and +40mV and reversed in direction at –60mV, positive of E_K (–78mV), suggesting that current flow was mainly carried by K^+. This I_{MTX} was blocked upon the addition

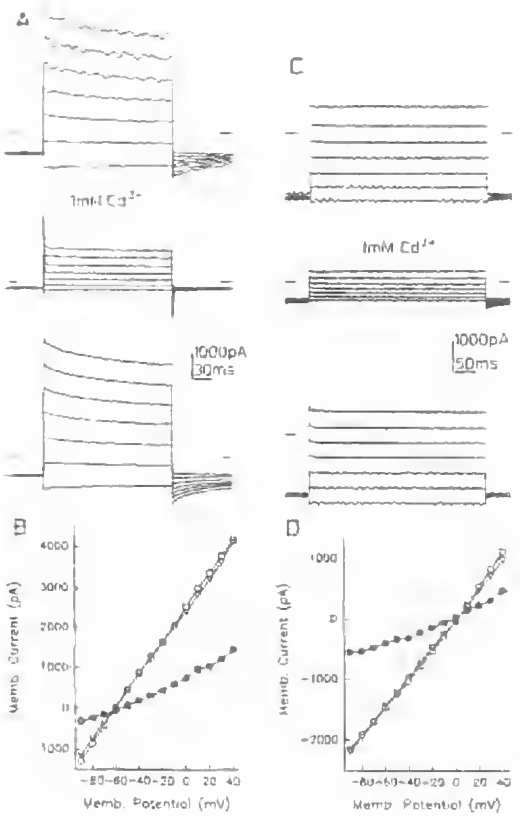

FIG. 3. MTX-induced currents in colonic cells recorded with K^+ or Cs^+-containing pipettes. MTX-induced whole-cell currents (I_{MTX}) every 20 mV between –90 and +30mV (from a holding potential of –80mV) in colonic cells filled with K^+-containing (A, B hollow circles) or Cs^+-containing (C, D hollow circles) pipette solutions. I_{MTX} was blocked by Cd^{2+} (1mM) (A,C middle panels; C,D filled circles), these effects of Cd^{2+} were quickly and readily reversible upon Cd^{2+} removal (A,C lower panels; B,D hollow triangles). Short lines on either side of these panels represent the zero current level. In *A,B*, the external Na^+ concentration had been mostly replaced with the impermeant TEA^+ (126mM), I_{MTX} reversed in direction near –60mV. In Cs^+-filled cells I_{MTX} reversed in direction near 0mV (C,D).

of Cd^{2+} (1mM) to the bathing solution (Fig.3A,middle panel; B). These effects of Cd^{2+} were completely reversible upon its removal (Fig.3A,B,lower panels; C,D). When the intracellular K^+ (126 mM) is replaced with Cs^+ (Fig.3C), a cation known to be permeant through many Ca^{2+} and non-selective cationic channels, but mostly impermeant through K^+ channels (Hille,1984), the reversal potential of I_{MTX} was near 0mV (Fig.3D), a reversal potential 10 to 20mV positive of the reversal potential obtained when K^+ was the main intracellular monovalent cation. The data suggest that I_{MTX} under normal physiological gradients is carried by Na^+ and K^+ and that Cs^+, but not TEA, will also freely pass through these MTX-induced channels

The channels activated by MTX may also allow the flow of, or be modulated by Ca^{2+}. The development of I_{MTX} was not prevented if Ca^{2+} in the bathing solution was omitted. However, I_{MTX} increased in amplitude if the Ca^{2+} concentration was raised to 6.25mM Ca^{2+}. I_{MTX} was also reduced but not blocked by nifedipine (1–10μM) or La^{3+} (1mM).

Effects of Internally-applied MTX

When MTX (5nM) was added to the pipette solution (containing 3mM EGTA) I_{MTX} developed slowly after formation of the whole-cell seal. Effects of MTX on the instantaneous whole-cell current (Fig.4B) activated by a ramp depolarization (to potentials between –60 and +100mV) showed this current reversed near 0mV and was sensitive to blockade by Cd^{2+} (0.1mM) (Fig.4C). This I_{MTX} was only 200–300pA in amplitude (at –60mV) compared with the 2000–3000 pA current (at –80mV) observed when MTX was applied externally (Fig.2). However, if the Ca^{2+} chelating agent, EGTA, was omitted from the pipette solution the I_{MTX} induced by internally applied MTX was larger and reversed at more negative potentials (near –60mV)(n=2). The addition of TEA (5mM) to bathing solution substantially reduced this I_{MTX} and shifted its reversal potential to near 0mV, suggesting that in the absence of EGTA the internal Ca^{2+} concentration is relatively high so that I_{KCa} contributes to the measured increase in current. These data also suggests that MTX can form/activate its channels from the internal surface of the membrane.

Single Channel Recordings

Recordings of the current flow through single MTX channels were made in the cell-attached patch clamp mode with patch pipettes filled with normal PS containing 10mM TEA and 5nM MTX. The membrane patches were depolarized with ramp depolarizations to obtain the instantaneous I-V relationship of any open channels. After seal formation the slope of the ramped-evoked current increased with time until discrete single channel openings and closings were observed. The current flow through these channels

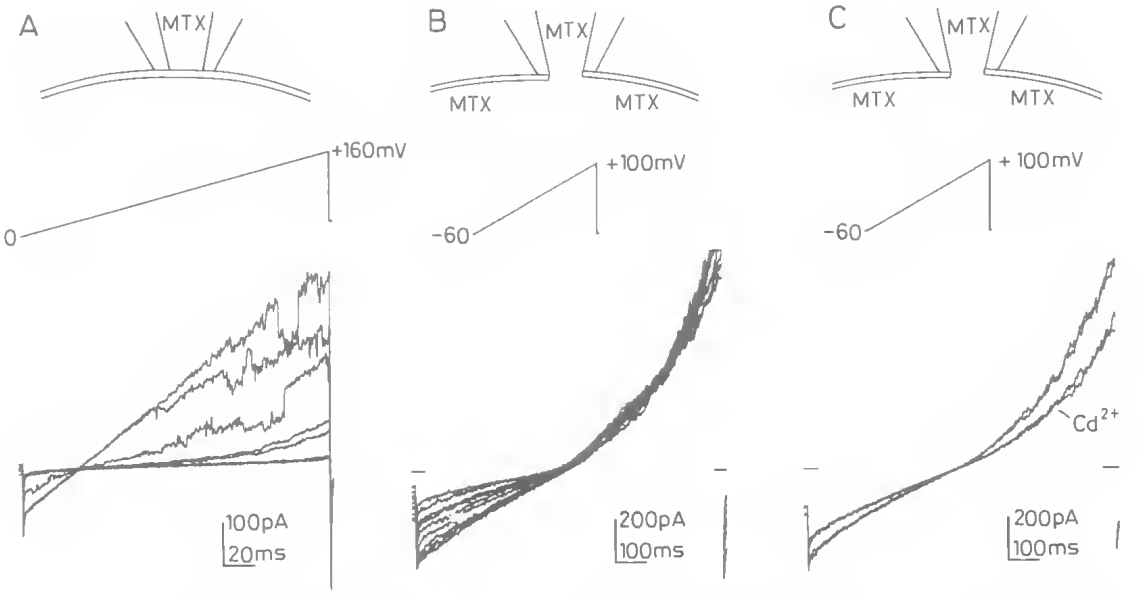

FIG. 4. Influence of pipette-applied MTX. A, cell-attached recordings of the development of MTX-activated single channels. Pipette solution contained normal saline plus 10mM TEA and 5nM MTX. B, development of whole-cell I_{MTX} after introduction of MTX (5nM) into the cell interior. Pipette solution contained high K^+ saline plus 3mM EGTA. I_{MTX} activated by internal MTX was also sensitive to extracellular Cd^{2+} (1mM) blockade (C).

was inward at the resting membrane potential (0mV added to the patch pipette). As the membrane patch was depolarized, however, the single channel amplitudes decreased until zero current flow was recorded at a potential some 30mV positive of the resting membrane potential. Outward current flow was recorded with further depolarization of the membrane patch. Such a reversal potential 30mV positive of the resting membrane potential is consistent with the reversal potential of I_{MTX} (–20 to –10mV) measured under whole-cell voltage clamp (Figs 2A,3A).

DISCUSSION

The spontaneous contractions of the circular muscle of the proximal colon and the contractions to acetylcholine were inhibited concentration-dependently by MTX (0.005-5nM). These effects of MTX followed an initial transient contraction to MTX (Fig.1) which was sensitive to blockade by the muscarinic antagonist, atropine. Given that MTX can stimulate rises in internal Ca^{2+} levels and neurotransmitter release from nerves and glands (Kim et al.,1985; Gusovsky et al.,1988), we suggest that this initial contraction arises from the release of acetylcholine from cholinergic motor neurones known to be present in this colon preparation. The subsequent blockade of the spontaneous activity and the contractions to acetylcholine were mimicked by monensin (0.1-10μM), the Na^+ ionophore, suggesting that a rise in the intracellular Na^+ is induced by MTX.

At the single cell level, MTX triggered a whole-cell inward current, I_{MTX}, that was some 50–100 times larger than the holding current (at –80mV) in control saline (Figs 2,3). The channels opened by MTX appeared equally permeable to K^+ and Na^+ as the reversal potential of I_{MTX} (–30 mV) was midway between E_K and E_{Na}. Confirmation of this reversal potential comes from the cell-attached single channel data which showed that the reversal potential of the single channel currents was some 30mV positive of the cell's resting membrane potential (likely to be –40 to –60mV) (Fig.4A). Replacing most of the external Na^+ with TEA^+ shifted the reversal potential of I_{MTX} to near –60mV, suggesting that, under these conditions, current flow was now mostly carried by K^+. Replacing the internal concentration K^+ with Cs^+ moved the I_{MTX} reversal potential some 20mV positive (to 0mV), even though it had a greater driving force (no added Cs^+ in the bath would mean that its Nernst potential would be very negative), suggesting that Cs^+ does not flow through these MTX channels as readily as K^+.

Preliminary calculations from the amplitudes of current flow through the MTX channels with voltage suggest that these channels have a conductance of up to 100pS. As MTX (5nM) activated an inward current of about 2000–3000 pA (at –80mV), this suggests that 330–500 channels/cell were activated. These channel currents do not arise from the recruitment of normal voltage-operated 'L-type' Ca^{2+} channels since the time course of I_{Ca} (or any of the K^+ currents) activated by membrane depolarization was not altered during the development of I_{MTX} (Yoshii et al.,1987); concentrations of nifedipine (10μM) which would block I_{Ca}, only slightly reduced I_{MTX}, and the reversal potential of I_{MTX} was approximately 50mV negative of the reversal potential of I_{Ca} in Cs^+-filled cells (Vogalis et al.,1993). It has been suggested, however, that MTX may well modulate Ca^+ channels, removing their voltage sensitivities for activation and inactivation and their ionic selectivity (Kobayashi et al.,1987; Yoshii et al. 1987). Perhaps a more attractive hypothesis is that MTX is triggering the opening of cation-selective channels normally opened by acetylcholine. These channels allow the flow of small cations and Ca^{2+}, have a reversal potential near 0mV and are opened by muscarinic agonists which stimulate phosphoinositide hydrolysis and IP_3-induced release of stored Ca^{2+} (Sims,1992). These channels, however, show a marked outward rectification at negative potentials and have a single channel conductance of 20–25pS (Inoue et al., 1987). If MTX is indeed opening these channels it must also be modifying them, removing their rectifying properties and perhaps inducing some sort of 'channel clustering'. If this is correct, our results indicate that 4–5 cholinergic channels would be needed to form a 'single' conducting pore with a conductance of 100pS. Other explanations are that MTX is an ionophore, or that it induces a pore in association with a membrane protein not necessarily involved in ion conductance. The delay in the action of MTX may also suggests that several MTX might be acting cooperatively.

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